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Comparison of Methods for the Rapid Detection of Streptococcus Bovis from Water

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COMPARISON OF METHODS FOR THE RAPID DETECTION OF *STREPTOCOCCUS BOVIS* FROM WATER

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor Date

Head, Microbiology Department Date

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The pollution of water by fecal material presents a health hazard to cities obtaining water from streams and rivers since many pathogenic microorganisms, including the fecal streptococci, are associated with this material. Recent studies have furnished data indicating that the fecal streptococci are more accurate than coliform tests for evaluating the sanitary quality of water (Bartley and Slanetz, 1960; Middaugh et al., 1971).

The fecal streptococci are gram-positive cocci found in the intestines of man and animals (Breed et al., 1957). They have been further subdivided into the enterococcus and viridans streptococci which contain the Lancefield Group D antigen. The enterococci include Streptococcus faecalis and its varieties, while Streptococcus bovis and Streptococcus equinus comprise the viridans group. The fact that the fecal streptococci do not occur at sites away from fecal contamination makes these microorganisms ideal indicators of fecal pollution (Croft, 1959). Since S. bovis and S. equinus are associated with ruminants and not man, the distinction between human and animal pollution can be made (Cooper and Ramadan, 1955). Koupal (1970) was able to routinely isolate and enumerate S. bovis from water samples by improving the existing methods of isolation. Kohlhoff (1973) made further improvements in the materials and methods of isolating S. bovis from surface waters.

The purpose of this investigation is to compare and improve

the existing methods of isolating S. bovis from water. These improvements would include the development of a method more selective for S. bovis than previous isolation techniques (Middaugh et al., 1971).

LITERATURE REVIEW

Classification

Thiercelin in 1899 (cited by Sherman, 1937) first used the term "enterococcus" to mean gram positive diplococci in the intestines of man. Many reviews (cited by Sherman, 1937) including those by Bagger 1925, 1926; Dible 1921, 1929; and Demeter 1929 have been written on the enterococci but most of the significant contributions to our knowledge have been made by studies on individual species.

In 1906 Andrews and Horder (cited by Deibel, 1964) suggested seven groups of streptococci based primarily on morphology, fermentation ability, and growth characteristics in milk. The predominant streptococcus, isolated from human feces, was termed Streptococcus faecalis. Streptococcus equinus was also named by Andrews and Horder in 1906 (cited by Sherman, 1937), who found it to be the principal streptococcus in the intestine of the horse.

Winslow and Palmer in 1910 and Fuller and Armstrong in 1913 (cited by Sherman, 1937) observed that the prevailing type of streptococcus in the feces of the cow ferments raffinose, while the predominating forms from the intestines of man and the horse do not have this property. This organism was described as a new species and named Streptococcus bovis by Orla-Jensen in 1919 (cited by Sherman, 1937), who obtained it from cow feces and from milk which had been heated or incubated at high temperatures. S. bovis was found to be an active fermenter of arabinose, raffinose, starch, and usually inulin. Ayers and Mudge in 1923 (cited by Sherman, 1937) studied S. bovis and added

further information concerning its characteristics. Sherman and Stark in 1931 (cited by Sherman, 1937) confirmed the results of Orla-Jensen and of Ayers and Mudge and also paid especial attention to the temperature limits of growth. They noted as a particular characteristic of S. bovis its ability to hydrolize starch actively when tested by the starch agar method.

Streptococcus inulinaceus was described by Orla-Jensen in 1919 (cited by Sherman, 1937) as an independent species although he noted that it was closely related to S. bovis and might be considered as a variety of that species. S. inulinaceus does not ferment starch and arabinose substances which are actively fermented by typical S. bovis. Sherman and Stark in 1931 (cited by Sherman, 1937) found that the members of the "bovis group" which did not hydrolize starch also failed to ferment arabinose, while the fermentation of arabinose, appeared to be perfectly correlated with the hydrolysis of starch in their strains of typical S. bovis; their results therefore tended to add some validity to S. inulinaceus as an independent type, although it was suggested that it probably should be considered as a variety of S. bovis. Bergey's Manual 6th edition considered S. inulinaceus as identical to S. bovis. Bartley and Slanetz (1960) point out that S. inulinaceus was not recognized in Bergey's Manual 7th edition. They also point out that organisms resembling S. inulinaceus were sometimes found in large numbers in human feces, but these organisms have not been detected in significant numbers in sewage. This indicates their apparent loss of viability outside the intestinal tract.

Sherman (1937) divided the streptococci into four primary groups based on temperature limits of growth supplemented by other basic characteristics as Lancefield group, hemolysis, growth at 10 C and 45 C, growth in presence of 6.5% NaCl, pH 9.6, and 0.1% methylene blue, strong reduction, survival at 60 C for 30 minutes, and NH_3 from peptone.

The four divisions recommended by Sherman were published in Bergey's Manual 7th edition. These are the pyogenic (no growth at 10 C or 45 C), the viridans (no growth at 10 C, growth at 45 C), the enterococcus (growth at 10 C and 45 C), and the lactic (growth at 10 C, but not 45 C). The enterococci are markedly differentiated from other known species of streptococci by their ability to grow at both 10 C and 45 C, growth in 6.5% NaCl, at pH 9.6, and in 0.1% methylene blue. They also have strong reducing powers. S. bovis and S. equinus are members of the viridans streptococci which cannot grow at 10 C, in 6.5% NaCl, at pH 9.6 and cannot tolerate 0.1% methylene blue. They have weak reducing powers.

Sherman (1938) reported that all of the enterococci had shown positive reactions with the Lancefield group D sera. About one half of the S. bovis cultures tested gave definite positive reactions with group D serum. S. equinus, found in the intestine of the horse, shows some points of relationship to S. bovis but none of the S. equinus tested with group D serum gave positive reactions.

Shattock (1949) showed that S. bovis contained the group D antigen using improved techniques. Smith and Shattock (1962)

reported that S. equinus also belonged to the serological group D. Because S. equinus is similar in many respects to S. bovis, which also belongs to serological group D (Shattock, 1949), the physiological reactions of the two species were compared. S. equinus does not ferment lactose while S. bovis does. Most of the strains of S. equinus tested did not ferment trehalose, raffinose or inulin, nor hydrolyze starch. In contrast, no strain of S. bovis was negative in all of these tests. S. equinus, being unable to ferment lactose, gives no reaction in litmus milk whereas S. bovis produces acid, though this reaction may be slow (several days) to develop. Thus S. equinus and S. bovis form fairly distinct groups.

Deibel (1964) and Hartman, Reinbold, and Saraswat (1966) have pointed out in excellent reviews of the fecal streptococci that the group D streptococci may now be considered to include S. bovis and S. equinus viridans and Sherman's enterococcus division and that both of these divisions are designated as fecal streptococci.

Occurrence and Significance

Ayers and Mudge in 1923 (cited by Cooper and Ramadan, 1955) reported S. bovis as the predominating streptococcus in bovine intestines.

Suckling in 1943 (cited by Croft, 1959) stated that enterococci are present in feces and sewage and are not found in pure water and sites away from human and animal contact. He also reported that enterococci do not multiply outside the animal body in water or soil. These conclusions (cited by Croft, 1959) were confirmed by Mallmann

and Litsky in 1951, Leininger and McClesky in 1953 and Morris and Weaver in 1954.

Sherman (1937) reported that Streptococcus faecalis and S. liquefaciens types were rather common on plants. Mundt, Johnson, and Khatchikian in 1958 (cited by Geldreich et al., 1964) reported that "the enterococci group" was isolated from 58.5% of 106 plant samples including 63 plant species. Mundt in 1963 (cited by Geldreich et al., 1964) described the occurrence of enterococci on plants from the Great Smokey Mountains. Enterococci were obtained from 14.2% of 2,169 flowers and 3.4% of 440 samples of leaves, buds, shoots, fruits, and seeds, but no evidence of plant-specific species or variants of enterococci were found. Geldreich, Kenner, and Kabler (1964) reported the biochemical results from 646 streptococci isolated from vegetation and 226 cultures isolated from insects. The predominant group, Streptococcus faecalis, as defined by Sherman's criteria, constituted a majority of all strains from vegetation and insects.

Eaves and Mundt in 1960 (cited by Deibel, 1964) reported that the association of streptococci with insects was circumstantial. The enterococci in the insect digestive tract are transient residents, and their occurrence on the insect exterior is due to mechanical transfer.

Deibel (1964) concluded that there is a general agreement that enterococci are not native to the soil, and their presence in soil samples represents contamination from either animal or plant sources. In this environment, the enterococci are disseminated most probably via wind, rain, and insects.

Cooper and Ramadan (1955) reported that typical S. faecalis did not exist among streptococci derived from cow and sheep feces. They concluded that typical S. faecalis seems to indicate a human origin while a starch-positive S. bovis points very definitely to an animal origin. They also concluded that the high incidence of S. bovis, isolated from feces of human infants by Sharpe in 1948, was due to the incomplete sterilization of the cows milk before feeding.

Bartley and Slanetz (1960) reported that studies on the types of streptococci present in feces of human beings and animals indicated that it might be possible to distinguish between human and animal contamination in water. The predominant type of streptococcus in human feces was not always typical S. faecalis, but it is not found in the feces of most domestic animals. Thus, its presence in water would indicate contamination of human origin. The presence of S. bovis in a water sample would indicate recent bovine or ovine pollution. They concluded that millipore filtering procedures for fecal streptococci are more accurate than coliform tests for evaluating the sanitary quality of water. In an investigation on the fecal streptococci in the feces of human beings and domestic animals by Kenner, Clark, and Kabler (1960), the fecal streptococci were to include the enterococcus group, S. mitis-salivarius group, S. bovis group, S. equinus group, and an atypical group closely resembling the enterococcus group. They reported that the median density of streptococci in millions per gram of moist feces was

1.3 for cow, 3.0 for human beings, 3.4 for fowl, 38.0 for sheep, and 84.0 for the pig. Enterococci densities were 0.16, 2.29, 2.10, 9.42, and 8.40 millions per gram, respectively. The average number of streptococci discharged in a 24-hour period increased in order of human being, fowl, cow, sheep, and pig. The enterococcus and S. salivarius groups predominated in human feces to account for 92.6% of the streptococcal population; enterococcus and streptococcal biotypes represented all the streptococci in feces of fowl; and quadrupeds showed appreciable quantities of the S. bovis and S. equinus groups, which were approximately 75% for cow, 46% for sheep, and 56% for the pig.

Cooper and Ramadan (1955) reported that Streptococcus faecalis was more frequently found in human feces and S. faecium was more predominantly found in animals. Studies using the heat-tellurite test developed by Ramadan and Sabir (1963) were run on fecal streptococci isolated from human and animal feces and frozen meat and poultry pies by Tilton and Litsky (1967). S. bovis, S. faecalis and S. fecium were isolated from these sources but an attempt to relate the presence of such organisms to their fecal source using the heat-tellurite test was unsuccessful.

Attempts to isolate S. bovis from a cattle lagoon by Iwami in 1967 (cited by Koupal, 1970) were unsuccessful. Geldreich and Kenner in 1969 (cited by Koupal, 1970) isolated S. bovis infrequently and in low numbers from river water. Koupal (1970) was able to routinely isolate S. bovis from river water by using his modified medium and method.

Starch Hydrolysis

The systematic application of the starch hydrolysis test in the classification of streptococci was first used by Andrews in 1930 (cited by Dunican and Seeley, 1962) to distinguish between hemolytic and nonhemolytic species from human infection. Sherman and Stark in 1931 (cited by Dunican and Seeley, 1962) found this characteristic to be of special importance in the identification of S. bovis which actively hydrolyzes starch. Sherman (1937) reported that S. bovis was very active in hydrolyzing starch, while S. equinus could do so only under favorable conditions. Seeley and Dain (1960) found that starch hydrolyzing streptococci fitting the description of S. bovis appeared to be widely spread in the alimentary tract of exotic as well as common domesticated ruminants. Dunican and Seeley (1962) reported that S. equinus did not hydrolyze starch under anaerobic conditions. This shows that S. equinus cannot liberate glucose from starch to initiate growth. All strains of S. bovis were shown to hydrolyze starch and initiate growth under anaerobic conditions when no glucose was present. These results indicate that the starch is not being broken down to the level of reducing sugars by S. equinus. Hobson and MacPherson (1952) reported that a streptococci (resembling S. bovis) isolated from the rumen of sheep split starch by an extracellular constitutive rather than an induced enzyme. This was substantiated by Dunican and Seeley (1952) who showed that cell free extracts of S. bovis could break down starch. Dunican and Seeley (1962) concluded that S. bovis has a starch-splitting enzyme of the alpha type based on their results and

those of Hobson and MacPherson in 1952. S. equinus seems to have a starch-splitting enzyme of the alpha-amylase type in that starch is broken down only to the level of oligosaccharides and not reducing sugars as is generally the case. In this respect, the enzyme from S. equinus seems to resemble the non-saccharifying type of alpha-amylase in Bacillus subtilis described by Kneen and Breckford in 1946 (cited by Dunican and Seeley, 1962), while the enzyme of S. bovis has the properties of the saccharifying alpha-amylase of B. subtilis.

Mann et al. (1954) reported finding a starch-fermenting strain of S. faecalis in the rumen of a sheep. Cooper and Ramadan (1955) also reported finding a starch-hydrolyzing S. faecalis which they designated as atypical faecalis IV. Of the 29 strains of fecal streptococci isolated from human feces, none were classified as atypical faecalis IV. Ramadan and Sabir (1963) reported that atypical faecalis IV was present only in the feces of buffalo, cow, camel, horse, and donkey and accounted for only 7.5% of the 361 strains studied. Like S. bovis, this group displays hydrolysis of starch and raffinose fermentation. Geldreich, Kenner, and Kabler (1964) reported that of the 646 strains of enterococci isolated from plants, 241 (37.7%) hydrolyzed starch even though all other criteria were typical of the enterococcus group. These results concurred with those of Langston and Bouma (1960) in a study of microorganisms of grass silage. They found that a majority of the streptococci with S. faecalis characteristics hydrolyzed starch. Koupal (1970) reported that 8% of the starch hydrolyzing streptococci isolated

from polluted water and animal feces were atypical S. faecalis. Utilization of starch by the group D streptococci is summarized in Figure 1. No isolations of S. uberis or S. equinus, which are weak starch hydrolyzing streptococci found in the bovine rumen (Seeley and Dain, 1960), were made from water samples or from fecal samples.

Kohlhoff (1973) reported that low molecular weight starch-dye complexes, such as amylose-azure (Calbiochem) and (RBB) Remazolbrilliant Blue R-Merck prepared in the laboratory, gave the largest zones of hydrolysis in the shortest period of time at a concentration of 0.2%. A starch concentration of 0.2% was recommended by Pavlova, Litsky, and Francis (1971) as an optimum level for indication of starch hydrolysis.

Media Development

In 1918 Weissenbach (cited by Litsky et al., 1953) was one of the first to describe a selective medium for enterococci. Sterile filtered ox bile was used for the inhibitory agent. Bagger in 1926 (cited by Litsky et al., 1953) also used sterile ox bile with one percent peptone to grow fecal streptococci.

Potassium tellurite medium proved to be highly selective in concentrating streptococci from human and animal feces (97% of the specimens). The advantage of a concentration of 1:5000 potassium tellurite is that it helps to diminish Proteus vulgaris and fungi which are often predominant in animal feces. Fleming in 1932 (cited by Cooper and Ramadan, 1955) discovered that some proteus strains

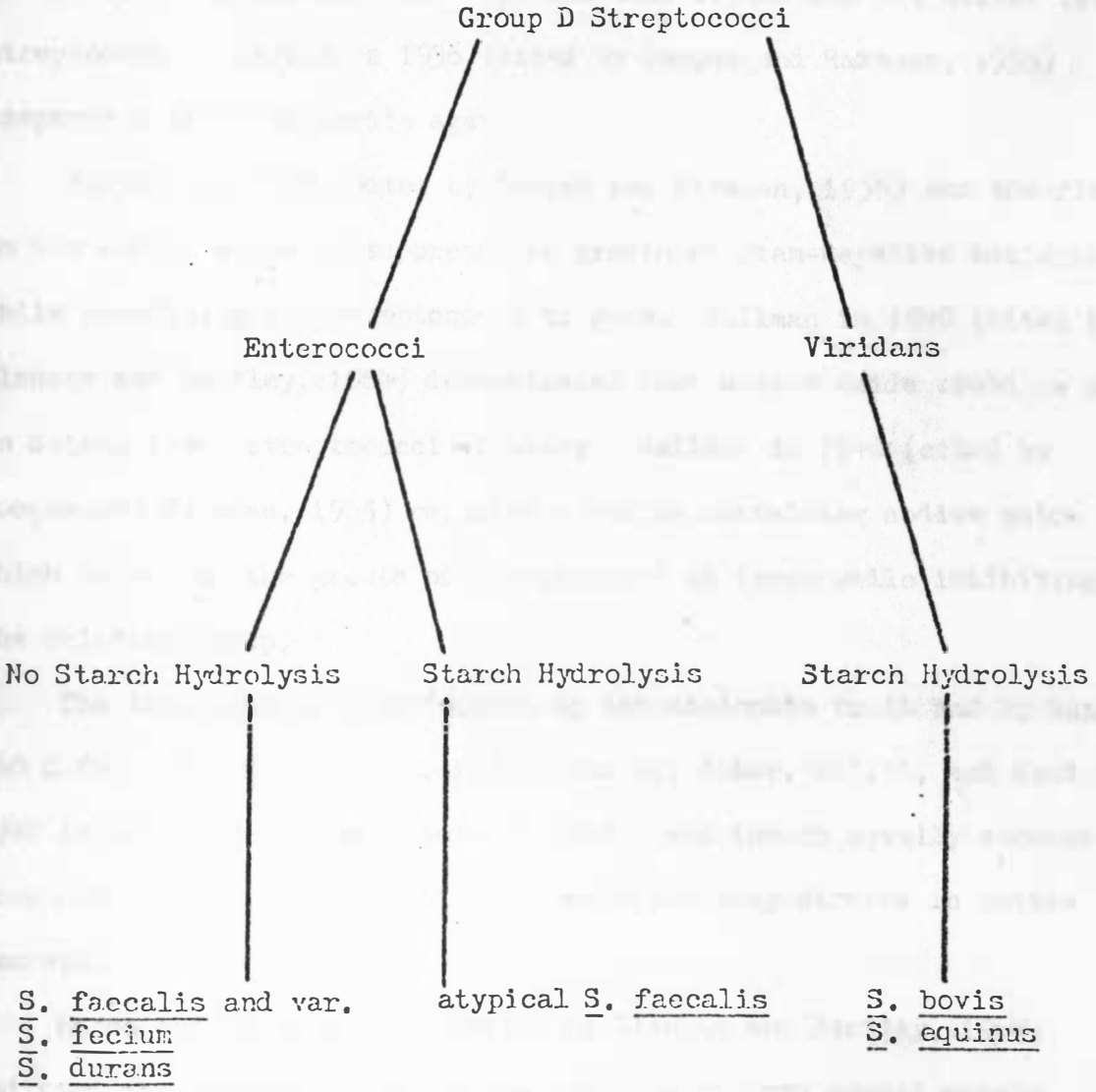


Figure 1. Utilization of starch by the group D streptococci.

resist 1:8000 potassium tellurite and that 1:5000 did not affect fecal streptococci. Harold in 1936 (cited by Cooper and Ramadan, 1955) prepared a solid tellurite agar.

Hartman in 1937 (cited by Cooper and Ramadan, 1955) was the first to use sodium azide to suppress the growth of gram-negative bacteria while permitting the streptococci to grow. Mallman in 1940 (cited by Slanetz and Bartley, 1964) demonstrated that sodium azide could be used to detect fecal streptococci in water. Mallman in 1940 (cited by Cooper and Ramadan, 1955) reported a medium containing sodium azide which supported the growth of streptococci in feces while inhibiting the coliform group.

The isolation of streptococci by tetrathionate broth and by heat (60 C for 1/2 hour) was compared by Cooper, Baker, Elliot, and Wood in 1942 (cited by Cooper and Ramadan, 1955), and though equally successful from human feces, showed that heat destroyed many strains in cattle excreta.

Hajna and Perry in 1943 (cited by Slanetz and Bartley, 1964) modified the Mallman medium by the addition of brom cresol purple.

Chapman in 1944 (cited by Litsky et al., 1953) published formulas of two media for isolation of streptococci. Tellurite streptococci medium contained crystal violet, trypan blue, and sodium tellurite as active agents. Approximately 3 per cent of the colonies grown on this medium were shown to be staphylococci. The azide violet blue medium contained S. T. 37, sodium azide, and crystal violet as inhibitory agents. As the sodium azide level was increased more coliforms as

well as the streptococci were inhibited. In 1946 Chapman (cited by Litsky et al., 1953) modified a previous medium for the isolation of enterococci which he called mitis-salivarius agar.

Winter and Sandholzer in 1946 (cited by Litsky et al., 1953) published a procedure for detecting the presence of enterococci. This method consisted of a sodium azide presumptive broth and a penicillin-methylene blue-sodium chloride broth slant confirmation medium.

Cooper and Linton in 1947 (cited by Cooper and Ramadan, 1955) showed the resistance of fecal streptococci to thallium acetate. Cooper and Ramadan (1955) reported that thallium acetate medium may be considered equal to tellurite medium in securing heavy pure growth of streptococci from animal feces but it failed in a considerable number of cases with human feces. Cooper and Ramadan (1955) and Ramadan and Sabir (1963) reported that a series of tests based on Janus green reduction, heat, and heat-tellurite tolerance could be used to differentiate between human and animal fecal streptococci. They concluded that a streptococcus which is capable of surviving the heat-tellurite test, regardless of its action on Janus green, may be regarded of human origin and one which failed to survive the heat-tellurite test and did not reduce Janus green could be considered of animal origin.

Rothe in 1948 (cited by Mallmann and Seligman, 1950) proposed a formulation for a sodium azide medium which was a modification of the medium reported by Mallman, Hajna, and Perry.

Mallman and Seligman (1950) made a comparative study on the available streptococcal media to determine the value of each. The media used in this study were standard lactose broth, Mallman's sodium azide broth, Hajna and Perry's S.F. broth, and Rothe's azide dextrose broth. In a series of tests, river water samples were checked for streptococci. The results showed that azide dextrose broth gave a higher index of growth on practically every sample. It was found however that gram-positive rods occurred in some of the tubes of azide dextrose broth from a few samples of river water. Quite frequently gram-positive rods appeared in S.F. broth and Mallman's azide broth. These results showed that azide dextrose broth was the best medium of those tested for the quantitative determination of streptococci from water.

In 1951 Hajna (cited by Kenner et al., 1961) modified the S.F. medium of Hajna and Perry. This new medium was named buffered azide glycerol glucose (BAGG) broth. Hajna (cited by Croft, 1959) described his BAGG broth as an improvement over his S.F. broth and he recommended it as a combined presumptive and confirmatory medium.

Litsky, Mallman, and Fifield (1953) developed a medium that was selective for the enterococci. This medium contained 0.04% sodium azide which the enterococci could tolerate and which inhibited gram-negative bacteria. Ethyl violet at a concentration of 0.00012 grams per liter inhibited gram-negative spore formers.

Barnes (1956) developed a medium which he called TG medium. This medium contained 2:3:5 triphenyl tetrazolium chloride at a

concentration of 375 micro-grams per 5 ml of medium. S. bovis was shown to have the least reducing powers against this chemical while S. faecalis and S. fecium had a greater ability.

Slanetz and Bartley (1957) improved the composition of the medium developed by Slanetz, Bent, and Bartley in 1955. This new medium, M-Enterococcus Agar, was shown to be 100 percent selective for enterococci.

Kenner, Clark and Kabler (1960) showed that EVA broth is inhibitory to S. bovis. Kenner, Clark, and Kabler (1961) developed a new medium KF (Kenner Fecal) streptococcus medium, and they showed that more strains of S. bovis could grow on this medium than on all others tested.

Cooper and Ramadan (1955) and Ramadan and Sabir (1963) reported that a series of tests based on Janus green reduction, heat, and heat-tellurite tolerance could be used to designate between human and animal fecal streptococci. They concluded that a streptococcus which is capable of surviving the heat tellurite test, regardless of its action on Janus green, may be regarded of human origin and one which failed to survive the heat-tellurite test and did not reduce Janus green could be considered of animal origin. Tilton and Litsky (1967) reported that fecal streptococci isolated from human and animal feces and frozen poultry and meat pies could not be catagorized into human or animal sources by the heat-tellurite method.

Tiede (1968) demonstrated that lowering the sodium azide level in M-enterococcus agar from 0.04% to 0.02% increased the recovery of

S. bovis while still inhibiting coliforms and spore formers. Koupal (1970) developed a modified M-enterococcus medium in which raffinose was substituted for glucose, and the sodium azide level was reduced from 0.04% to 0.02%. The azide level of 0.02% was still sufficient to inhibit coliforms and spore formers without being inhibitory to S. bovis.

Wolin, Manning, and Nelson (1959) reported the ability of S. bovis to utilize NH_4Cl as the sole source of nitrogen. McCoy and Wender (1953) showed that S. faecalis requires ten amino acids for growth. Koupal (1970) attempted to use the NH_4Cl utilizing ability of S. bovis to prepare a selective medium in which S. bovis could grow but S. faecalis and the other enterococci would be inhibited. He showed that NH_4Cl was inferior to tryptose as a nutrient. A concentration above 0.02% was toxic to S. bovis.

Kohlhoff (1973) reported that Koupal's modified M-enterococcus medium was only slightly less inhibitory to S. bovis even though the sodium azide was decreased by fifty percent. He also developed a modified KF streptococcus medium which was less inhibitory to S. bovis. A sodium azide level of 0.02% and a sodium chloride level of 0.1% was more suited for the growth of S. bovis. He showed that raffinose was not a more suitable carbon source than maltose or dextrose.

Rochaix in 1924 (cited by Facklam and Moody, 1970) called attention to the value of esculin hydrolysis in the identification of enterococci. The enterococci were able to split esculin, but

other streptococci could not. Isenberg, Goldberg, and Sampson (1970) noted that the combination of esculin and a rather low concentration of bile in the presence of 0.025% sodium azide in Pfizer Selective Enterococcus (PSE) agar permits the selection of enterococci and other group D streptococci by typical colonial morphology and visible evidence of esculin hydrolysis which was not observed by any other bacterium tested. All enterococci and related streptococci grew within 18 to 24 hours as round, entire, convex, translucent to whitish colonies, 1 to 2 mm in diameter, surrounded by dark brown-to-black appearing iron salts.

Sabbaj, Sutter, and Finegold (1971) reported that all of the group D streptococci including S. bovis grew on Pfizer Selective Enterococcus (PSE) agar. All of the strains of S. bovis tested by Lee (1972) grew on (PSE) agar. Pavlova et al. (1972) found that (PSE) agar had high counts of fecal streptococci and low counts of nonfecal streptococci in tests conducted with fecal streptococci and other bacteria.

Isolation Techniques

Litsky, Mallman, and Fifield (1953) developed an MPN (Most Probable Numbers) procedure for the enumeration of enterococci in water. They also proposed a new test for enterococci where glucose azide broth is used as a presumptive medium and ethyl violet azide broth as a confirmatory medium.

The membrane (molecular) filter (MF) was first described in this country by Goetz in 1947 (cited by Kabler, 1954). Clark,

Geldreich, Jeter, and Kabler in 1951 (cited by Kabler, 1954) recommended specific methods and materials for use with the membrane filter in sanitary bacteriology. Kabler (1954) investigated the results of water examinations by the MF technique with those obtained simultaneously by the Standard Methods fermentation tube (MPN procedure) given in the 9th edition of Standard Methods for the Examination of Water and Sewage. The results indicated that the two test procedures did not measure the same group of organisms and that the sanitary significance of the differences in the results was yet to be determined.

Slanetz, Bent, and Bartley (1955) developed a membrane filter technique for the detection and enumeration of enterococci in water which was similar to the technique used for detecting coliforms in water. Various media and inhibitory substances were tested for use with the membrane. To color the colonies 0.01% 2,3,5 tetrazolium chloride (TTC) was added to the media. The ability of unnamed strains of fecal streptococci to reduce tetrazolium was reported by Laxminarayana and Iya in 1953 (cited by Barnes, 1956). The colonies as reported by Slanetz et al. (1955) were either light pink colonies or raised, glistening, dark red colonies with a pink periphery. At the end of 48-hour incubation at 35 to 37 C, the colonies ranged in size from 0.5 to 2.0 mm in diameter. Of all of the media tested, a modification of the Chapman Mitis-Salivarius medium proved to be the most satisfactory for the cultivation of enterococci on the filters. This membrane filter technique showed that the enterococci could be

used to test the sanitary quality of water and foods more reliably than coliform organisms.

Barnes (1956) showed that the amount of tetrazolium reduction could be used to differentiate S. faecalis and S. fecium from S. bovis. Wiebe, Raj, and Liston (1961) reported that S. bovis and S. durans gave white colonies on tetrazolium agar. Tilton and Litsky (1967) reported that cultures of S. bovis gave brick red or colorless colonies on medium containing TTC.

Slanetz and Bartley (1957) found that the membrane filter technique gave higher counts of enterococci than the MPN procedure developed by Litsky et al. in 1953. Based on the arithmetic mean counts, the ratio of enterococci to coliforms was 1.9:1 for water samples, 1:1.7 for sewage samples, 1:1.6 for fecal samples from humans, and 15:1 for fecal samples from animals. This showed that the detection of enterococci would be the most efficient method for determining the sanitary quality of water and other materials.

Sureau in 1958 (cited by Slanetz and Bartley, 1964) reported that the M-Enterococcus Agar developed by Slanetz and Bartley gave satisfactory results for the isolation and enumeration of fecal streptococci in water samples tested by the membrane filter technique and he noted that this procedure had been adopted by the Pasteur Institute in Madagascar for the routine bacterial analysis of water. Catsaras in 1958 (cited by Slanetz and Bartley, 1964) also reported favorable results with M-Enterococcus Agar using membrane filter procedures.

Croft (1959) ran a comparative study on the methods used for the detection of enterococci in water. The Azide Dextrose (AD)-Ethyl Violet Azide (EVA) tube dilution method of Litsky and the membrane filter method using M-Enterococcus Agar developed by Slanetz and Bartley (1964) gave the same order of productivity and were included for the detection of fecal streptococci in the 11th edition (1960) of Standard Methods for the Examination of Water and Wastewater.

Kenner, Clark, and Kabler (1961) demonstrated that KF streptococcus medium used as a MPN test or as a membrane filter procedure yielded higher results in the recovery of streptococci than the M-Enterococcus membrane filter method or the AD-EVA MPN method. Slanetz and Bartley (1964) reported that KF streptococcus medium is not as suited for selective detection of fecal streptococci using the membrane filter technique as M-Enterococcus agar because it was found that KF streptococcus agar does not support the growth of S. bovis on membrane filters.

Koupal (1970), using the starch-hydrolyzing ability of S. bovis, developed a starch-agar-iodine layer method to isolate this organism from polluted water. Millipore filtering techniques (APHA Standard Methods, 1965) were used to isolate S. bovis. A 1% soluble starch layer was placed on top of the membrane and the plates were then incubated anaerobically at 37 C for 24 to 48 hours. The plates were then flooded with 1% aqueous iodine to show the zones of hydrolysis. However, 8% of the starch hydrolyzing group D

streptococci were shown to be atypical S. faecalis, a starch hydrolyzing enterococcus as described by Mann et al. 1954 and Cooper and Ramadan 1955.

Lee (1972) investigated the reliability of 2 new media in differentiating group D enterococci from other streptococci. These two new media were TD medium and D broth. One hundred percent of the strains of S. bovis and the enterococci grew on Pfizer Selective Enterococcus (PSE) agar but none of the S. bovis strains grew in either TD medium or D broth while 95% of the enterococci grew in these media. Using these results, S. bovis could be differentiated from the enterococci.

Kohlhoff (1973) studied the use of dye-labelled starches to omit the iodine step used by Koupal. He used the Rinderknecht (1967) method of employing dye labelled starches into the substrate. Remazolbrilliant Blue R (RBB) was attached to Merck hydrolyzed starch. This starch-dye complex along with amylose azure and amylopectin azure were compared as indicators of starch hydrolysis. A medium containing 0.2% starch-dye complex placed on top of a membrane filter containing S. bovis and then incubated for 24 to 48 hours under anaerobic conditions showed that starch-dye complexes could be used to show starch hydrolysis as indicated by clear zones around the colonies in the colored medium.

MATERIALS AND METHODS

Source of Cultures

The known stock cultures of fecal streptococci for this study were obtained from four sources: the American Type Culture Collection (ATCC); Microbiology Department, South Dakota State University (SDSU); isolations from bovine feces from the SDSU Dairy Farm; and Dr. R. R. Facklam of the Center for Disease Control (CDC) laboratory at Atlanta, Georgia. Streptococcus faecalis and Streptococcus bovis were obtained from the ATCC with code numbers 8043 and 9809, respectively. Cultures of Streptococcus faecalis, Streptococcus faecalis var, liquefaciens, and Streptococcus equinus were obtained from the culture collection at SDSU. Known cultures of Streptococcus bovis were obtained from the CDC laboratory. Additional cultures of Streptococcus bovis were isolated from fresh bovine feces from the SDSU dairy unit.

The stock cultures used in this study were maintained by inoculating the organisms into a stock culture maintenance medium described by Tiede (1968) as given below:

Maintenance Medium

Bacto tryptose	20.0 g
Yeast extract	5.0 g
Glucose	1.0 g
Dipotassium phosphate	4.0 g
Bacto agar	10.0 g
Water	1000.0 ml
pH	7.2

The organisms were incubated for 24 hr at 35 C and then stored at 4 to 6 C.

Stock Cultures

Preparation of cultures for all experiments followed the same procedure. A 3 mm loop was used to transfer known culture cells from the maintenance medium into sterile Brain Heart Infusion (BHI) broth (Difco Laboratories). The broth tubes were then incubated at 35 C for 19 hr in a Torbal Jar (Model AJ-2, The Torsion Balance Co.) which maintained an atmosphere of 75% N_2 and 25% CO_2. This procedure produced a cell concentration of approximately 15 $X10^8$ organisms/ml using membrane filter procedures (APHA Standard Methods, 1971) with modified KF Streptococcus (KF) medium (Kohlhoff, 1973). Colonies were counted using an American Optical binocular stereoscope at a magnification of 20X.

Plate Count Media

The plate count media used in this study were tested for their ability to enhance the growth of S. bovis. These media include modified M-Enterococcus medium (Koupal, 1970), modified KF Streptococcus medium (Kohlhoff, 1973), and Pfizer Selective Enterococcus (PSE) agar. Plate counts were also made on modifications of these media.

M-Enterococcus medium was developed for the isolation of enterococci using membrane filter techniques (Slanetz and Bartley, 1957). Tiede (1968) lowered the sodium azide level from 0.04% to 0.02%, and Koupal (1970) substituted raffinose for dextrose.

Reducing the sodium azide concentration made the media less inhibitory to S. bovis while still inhibiting the growth of gram negative bacteria and spore formers. Kohlhoff (1973) demonstrated that raffinose substituted for dextrose gave only slightly better results. He reasoned that this was probably due to raffinose being a nonreducing trisaccharide which is difficult to ferment, while dextrose, being a monosaccharide, is more readily utilized by S. bovis. The medium was described in the following manner:

Modified M-Enterococcus Medium

Bacto tryptose	20.0 g
Yeast extract	5.0 g
Dipotassium phosphate	4.0 g
Raffinose	2.0 g
Sodium azide	0.2 g
Bacto agar	10.0 g
TTC	10.0 ml
Water	1000.0 ml

Kenner, Clark, and Kabler (1961) developed KF (Kenner Fecal) Streptococcus medium. They demonstrated that this medium yielded higher results in the recovery of fecal streptococci than the M-Enterococcus medium using membrane filter procedures. Slanetz and Bartley (1964) reported that KF Streptococcus agar did not support the growth of S. bovis on membrane filters. Kohlhoff (1973) modified the KF medium to make it less inhibitory to S. bovis. The first modification was the reduction of sodium azide from 0.04% to 0.02% and the elimination of Bacto-brom cresol purple. Koupal (1970) found that the chloride ion inhibited growth of S. bovis. Kohlhoff found that reducing the NaCl concentration in the KF medium from

0.5% to 0.1% promoted the growth of S. bovis to about the same extent as did reducing the sodium azide content. Raffinose in place of maltose limited growth to a much greater extent than did sodium azide or sodium chloride so maltose, which is fermented by both S. bovis and S. faecalis (Breed et al., 1957), was used in the modified KF medium. The combination of these modifications is given in the following formula:

Modified KF Streptococcus Medium

Bacto proteose peptone No. 3----------	10.0 g
Yeast extract----------	10.0 g
Sodium chloride----------	1.0 g
Sodium glycerophosphate----------	10.0 g
Maltose----------	20.0 g
Sodium azide----------	0.2 g
Bacto agar----------	10.0 g
TTC----------	10.0 ml
Water----------	1000.0 ml

Pfizer Selective Enterococcus (PSE) agar, only recently available commercially, was prepared by following the enclosed directions. This medium contains 0.025% sodium azide as an inhibitory agent and esculin, hydrolyzed only by "fecal" streptococci (Facklam and Moody, 1970), as a selective ingredient. Isenberg, Goldberg, and Sampson (1970) noted that the combination of esculin and a low concentration of bile permits the selection of enterococci and other group D streptococci. Lee (1972) reported that all strains of S. bovis grew on PSE agar.

The modification of the previously modified KF Streptococcus medium was the elimination of maltose. The reasoning behind this elimination is that if starch-dye complexes are introduced into

the medium, S. bovis is the only active starch-hydrolyzing viridans streptococcus which could initiate growth with starch substituted as the major carbon source (Dunican and Seeley, 1962). The enterococci, with the exception of atypical faecalis, cannot hydrolyze starch (Mann et al., 1954; Cooper and Ramadan, 1955; Ramadan and Sabir, 1963; and Geldreich, Kenner, and Kabler, 1964). The starch-dye complex used was amylose azure (Calbiochem Cat. No. 17266, 1974). Kohlhoff (1973) reported that amylose azure was the substrate most susceptible to the alpha amylase produced by S. bovis. The maltose was eliminated and the resultant medium was designated as "KFO1". KFO1 is given in the following formula:

KFO1 Medium

Bacto proteose peptone No. 3----------	10.0 g
Yeast extract----------	10.0 g
Sodium chloride----------	1.0 g
Sodium glycerophosphate----------	10.0 g
Sodium azide----------	0.2 g
Bacto agar----------	10.0 g
TTC----------	10.0 ml
Water----------	1000.0 ml

The elimination of esculin was made in PSE agar. This medium was designated as "modified PSE agar" and is given in the following formula:

Modified PSE Agar

Pfizer peptone C----------	17.0 g
Pfizer peptone B----------	3.0 g
Pfizer yeast extract----------	5.0 g
Pfizer bacteriological bile----------	10.0 g
Sodium chloride----------	5.0 g
Sodium citrate----------	1.0 g
Ferric ammonium citrate----------	0.5 g
Sodium azide----------	0.25 g

Pfizer agar	15.0 g
TTC	10.0 ml
Water	1000.0 ml

All of the media except PSE agar contained filter sterilized 2,3,5-triphenyl tetrazolium chloride (TTC) to color the colonies (Slanetz, Bent, and Bartley, 1955). TTC was added at a concentration of 1.0 ml 1.0% TTC per 100 ml of medium. All media used for plate count procedures and other growth studies were autoclaved at 121 C and 15 psi for 10 minutes.

Selective ingredients of these media are summarized in Table 1.

Plating Techniques

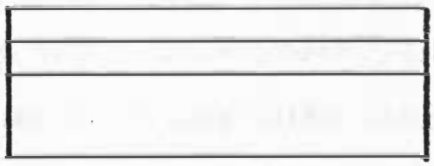
Three different plating techniques were evaluated in this study for their ability to show starch hydrolysis by Streptococcus bovis (Figure 2). These procedures are the starch-agar-iodine overlay method developed by Koupal (1970), the starch-dye-medium method developed by Kohlhoff (1973), and the starch-dye-agar overlay method, a modification of Koupal's method.

Sherman (1937) reported that the ability of S. bovis to hydrolyze starch was an important characteristic when identifying this microorganism. Koupal (1970) relied upon this characteristic to develop a membrane filter technique to isolate and enumerate S. bovis from fresh water, fecal swabs from cattle and swine, and known cultures. Ten ml amounts of sterile modified M-Enterococcus medium were dispensed into sterile 60 mm petri dishes. The membrane filter technique was used to isolate and enumerate S. bovis from the samples (APHA Standard Methods, 1965). The samples were filtered through

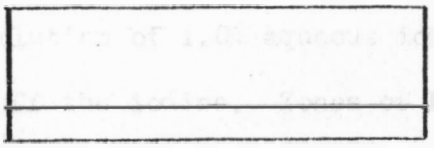
Table 1. Selective ingredients of selective enterococcal media.

Medium	NaCl Concentration (%)	NaN_3 Concentration (%)	Sugar (%)
M-Enterococcus	0.0	0.04	Dextrose 0.2
Modified M-Enterococcus	0.0	0.02	Raffinose 0.2
KF Streptococcus	0.5	0.04	Maltose 2.0
Modified KF Streptococcus	0.1	0.02	Maltose 2.0
Pfizer Selective Enterococcus	0.5	0.025	Esculin 0.1

Starch-Agar-Iodine Overlay



Starch-Dye-Medium



Starch-Dye-Agar Overlay

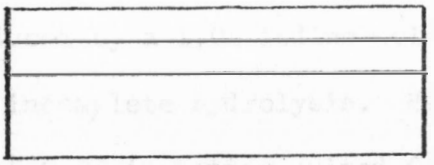


Figure 2. Methods for isolating Streptococcus bovis.

Millipore HAWG filters. After filtration and placement of the membrane filters on the desired medium, 1.5 ml of sterile starch agar was aseptically layered over the membrane. The starch agar contained 1.0% soluble starch (Fisher Scientific Co., indicator grade), and 0.75% agar (Difco Laboratories). After the starch-agar layer was allowed to solidify, the membrane plates were incubated at 37 C in an atmosphere of 25% carbon dioxide and 75% nitrogen for 24 to 48 hr. Enumeration of the streptococci which hydrolyze starch was accomplished by flooding the starch-agar layer on the membrane with a solution of 1.0% aqueous iodine for two minutes before pouring off the iodine. Zones of hydrolysis ranging in size from 3 to 5 mm in diameter allowed an accurate count of the numbers of starch-hydrolyzing colonies. Plates with 50 or more zones were difficult to count because of the confluence of the zones.

Kohlhoff (1973) noticed that the dark blue starch-iodine complex produced by a 1.0% iodine solution obliterated the smaller zones of incomplete hydrolysis. He reported that an iodine concentration of 0.5% is better suited for Koupal's method of detecting starch hydrolysis. Kohlhoff also pointed out that after flooding the plates with iodine, further growth studies can no longer be continued, and that the fading of the starch-iodine complex makes it impossible to store a plate for further reference to a particular colony. An additional modification was made to Koupal's procedure. A 0.2% starch concentration was used. Pavlova, Litsky, and Francis (1971) showed this to be the optimum level for visual starch hydrolysis

by S. bovis.

The resultant procedure used in the course of this investigation takes place in the following steps:

1. A Millipore Standard Hydrosol Filter unit is initially sterilized by autoclaving for 15 min at 121 C and 15 psi.
2. The agar medium to be used is cooled to 45-55 C. Sterile wide mouth pipettes which had been pretempered to 45-55 C are used to layer 3 ml of medium into commercially sterile 60 mm petri dishes (Falcon Plastics, #1007 petri dish).
3. The agar medium is allowed to solidify.
4. The desired volume of water sample is filtered through 47 mm Millipore HAWG filters, and the filters aseptically transferred to the petri plates.
5. Two ml of 0.2% starch agar pretempered to 45-55 C is then placed upon the filters. The starch agar contained 0.2% soluble starch (Merck and Co. No. 09903) and 1.0% agar (Difco Laboratories).
6. The starch agar layer is allowed to solidify and the plates are not inverted, and are incubated at 35 C in an atmosphere of 75% nitrogen and 25% carbon dioxide maintained in a Torbal Jar (Model AJ-2, The Torsion Balance Co.). The relative humidity is kept near 100%, and a slight negative pressure is allowed for gas evolution by the bacteria.
7. After 24 hours the plates are flooded with 0.5% iodine solution which contained twice the amount of potassium iodide as the amount of iodine per 100 ml distilled water. Colonies are counted and

starch-hydrolysis zone size and sharpness measured with the aid of an American Optical binocular stereoscope at a magnification of 20X.

Rinderknecht et al. (1967) reported a new method for detecting starch hydrolysis employing a dye labelled starch as the substrate. The dye used for labelling the starch was Remazolbrilliant Blue R (RBB) (Calbiochem, #55435). The detection of amylase activity of a bacterial culture is determined colorimetrically. The dye is solubilized by the action of amylase and a clearing of the medium is observed.

Kohlhoff (1973) prepared a RBB-starch complex. This contained RBB and soluble starch (Merck and Co. #09903). A new membrane filter procedure was developed to compare the amylase activity of S. bovis on this starch-dye complex and other starch-dye complexes. The other starch-dye complexes evaluated were amylose azure and amylopectin azure (Calbiochem #17266 and 172678). Known samples of S. bovis were filtered and then these filters were placed in the bottom of 60 mm petri dishes. Three ml of the desired medium containing 0.2% starch-dye was placed on top of the filters. The plates were then incubated for 24 to 48 hr at 37 C in an atmosphere of 75% nitrogen and 25% carbon dioxide. Amylose azure was the substrate most susceptible to the enzyme alpha amylase as was demonstrated by the largest zones of hydrolysis in the shortest time of zone development; 4 to 6 mm zones were produced in 24 hr. Amylopectin azure, being of higher molecular weight than amylose

azure, was not broken down as readily and although the zones were sharper, they were not as large as those produced in media containing amylose azure. RBB-Merck gave zones intermediate in size to amylose azure and amylopectin azure. The starch-dye complex incorporated into the medium eliminated the iodine step in Koupal's method. The cultures can therefore be observed at any time and the plates stored for further reference.

The plating technique used in this study is a modification of that given by Kohlhoff (1973). The procedure developed takes place in the following steps:

1. A Millipore Standard Hydrosol Filter unit is initially sterilized by autoclaving for 15 min at 121 C and 15 psi.
2. The desired volume of water sample is filtered through 47 mm Millipore HAWG filters and the filters aseptically transferred to sterile 60 mm petri dishes (Falcon Plastics, #1007 petri dish).
3. The agar medium to be used is cooled to 45-50 C. Sterile wide-mouth pipettes which had been pretempered to 45-55 C are used to layer 3.0 ml of medium onto the filters.
4. The agar medium is allowed to solidify and the plates are not inverted, and are incubated at 35 C in an atmosphere of 75% nitrogen and 25% carbon dioxide maintained in a Torbal Jar (Model AJ-2, The Torsion Balance Co.). The relative humidity is kept near 100%, and a slight negative pressure is allowed for gas evolution by the bacteria.

5. The plates are examined at 24 and 48 hr. Colonies are counted and starch-hydrolysis zone size and sharpness measured with the aid of an American Optical binocular stereoscope at a magnification of 20X.

An additional method for enumerating S. bovis was developed in this investigation. This procedure is a modification of the starch-agar-iodine overlay procedure. A starch-dye-agar layer was substituted for the starch agar. This starch-dye agar contained 0.2% starch-dye and 1.0% agar (Difco Laboratories). Known cultures of S. bovis were incubated and counted as described in the previous section.

The starch-dye complexes used in this investigation were amylose azure and amylopectin azure (Calbiochem, #17266 and 172678).

RESULTS AND DISCUSSION

Tiede (1968), Koupal (1970), and Kohlhoff (1973) reported that the commercially available enterococcal media were inhibitory to Streptococcus bovis, a viridans streptococcus. Modifications were made to these media so that they supported the growth of S. bovis. These modifications, along with improved isolation techniques, made it possible to isolate and differentiate S. bovis from the non-starch-hydrolyzing fecal streptococci. Lee (1972) investigated the ability of S. bovis to initiate growth on Pfizer Selective Enterococcus (PSE) agar. He reported that one hundred percent of the strains of S. bovis studied were able to initiate growth on this medium. The present investigation was undertaken to compare PSE agar to the previously modified enterococcal media in its ability to enumerate S. bovis. The second phase of this investigation was to compare and improve the starch-hydrolysis methods of isolating S. bovis.

Comparison of Various Growth Media Using Membrane Filter Procedures

The main objective of these growth studies is to determine which of these media is most suitable for the enumeration of S. bovis.

The comparative results of plate counts of S. bovis on media used for membrane filtration of water are in Table 2. A 48 hr incubation period is required before enumeration of colonies can be made on modified M-Enterococcus agar as compared to 24 hr on

modified KF streptococcus agar. This additional 24 hr incubation period indicates that modified M-Enterococcus agar is not as suited for membrane filter procedures as is modified KF Streptococcus agar. This confirms the results obtained by Kohlhoff (1973) in a similar study on the membrane filter application of various media. Modified KF Streptococcus agar and PSE agar produce equal numbers of S. bovis using both amylose azure and Merck starch, low molecular weight starches, and amylopectin azure, a high molecular weight starch. These results disagree with those of Kohlhoff (1973) who found an eight-fold difference in plate counts between the low molecular and high molecular weight starches.

Starch-Agar-Iodine Overlay Method

The starch-agar overlay procedure developed by Koupal (1970) was used to compare the starch-hydrolysis zones produced by S. bovis on various media. The results obtained in this study indicate that PSE agar was superior to modified M-Enterococcus agar and modified KF Streptococcus agar for the visualization of starch-hydrolysis zones of S. bovis (Table 3). Brown-to-black appearing iron salts surrounding the colonies indicating esculin hydrolysis (Isenberg et al., 1970), make the zones clearer to distinguish than the clear zones produced on modified M-Enterococcus and modified KF Streptococcus agars. Typical zones of starch hydrolysis produced by S. bovis on modified KF Streptococcus agar employing this technique are shown in Figure 3.

Table 2. Summary of plate counts of Streptococcus bovis on various media.[a]

Medium	Starch (0.2%)	Type of Hydrolysis Indicator	No. of Bacteria/ml ($\times 10^8$)
Modified M-Enterococcus	Merck	Starch-agar overlay	14
Modified M-Enterococcus	Amylose azure	Starch-dye overlay	13
Modified KF Streptococcus	Merck	Starch-agar overlay	12
Modified KF Streptococcus	Amylose azure	Starch-dye medium	10
Modified KF Streptococcus	Amylose azure	Starch-dye overlay	10
Modified KF Streptococcus	Amylopectin azure	Starch-dye medium	10
Modified KF Streptococcus	Amylopectin azure	Starch-dye overlay	10
Pfizer Selective Enterococcus	Merck	Starch-agar overlay	12
Pfizer Selective Enterococcus	Amylose azure	Starch-dye medium	10
Pfizer Selective Enterococcus	Amylose azure	Starch-dye overlay	10
Pfizer Selective Enterococcus	Amylopectin azure	Starch-dye medium	9
Pfizer Selective Enterococcus	Amylopectin azure	Starch-dye overlay	9

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2; time, 24 hr with exception of modified M-Enterococcus which was 48 hr.

Table 3. Summary of starch-hydrolysis zones produced by Streptococcus bovis using the starch-agar-iodine overlay method.[a]

Medium	Sugar (%)	Starch Zone Size (mm)	Starch Zone Sharpness[b]
Modified M-Enterococcus	Raffinose 0.2	1.50	+
Modified KF Streptococcus	Maltose 2.0	3.00	++
Pfizer Selective Enterococcus	Esculin 0.1	5.00	+++

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2; time, 24 hr; hydrolysis indicator, 0.2% hydrolyzed Merck starch-0.5% aqueous iodine.

[b] A rating of (+) is the lowest rating and (+++) the highest rating.

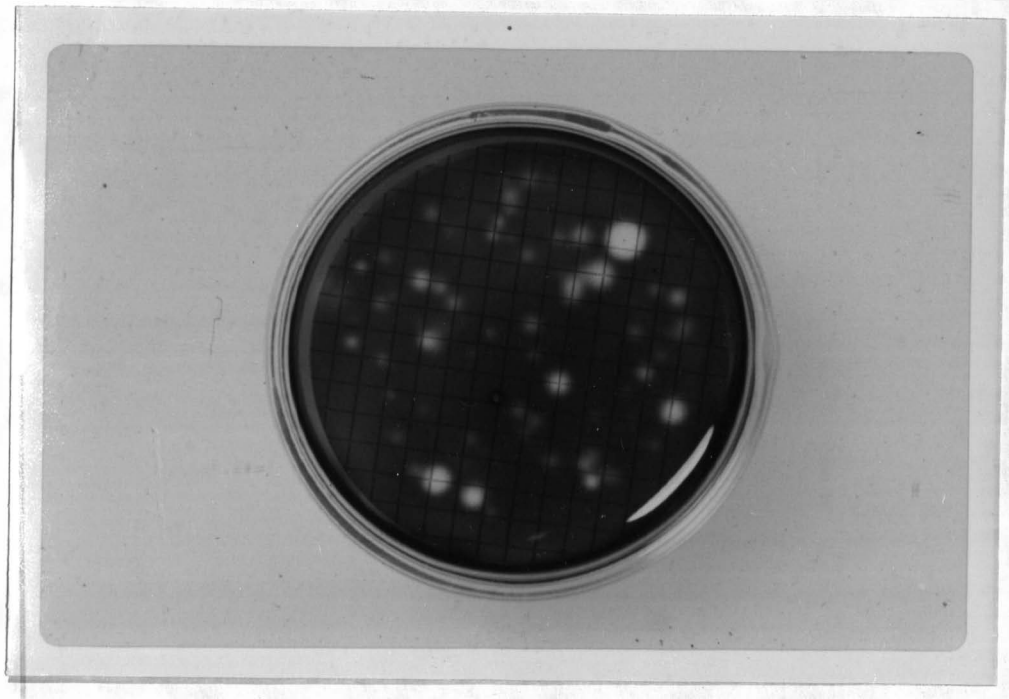


Figure 3. Zones of starch hydrolysis produced by Streptococcus bovis on a membrane filter layered with 0.2% starch agar.

Starch-Dye Medium Method

The isolation technique developed by Kohlhoff (1973) can be used to visualize starch hydrolysis with starch-dye complexes, thus eliminating the addition of iodine. The starch-dye complexes used in this study are those described in Materials and Methods. Table 4 provides a summary of data concerning the starch-hydrolysis zones produced using various media and starch-dye complexes. KFO1 agar plus amylose azure gave the largest zones of hydrolysis. It may be speculated from these results that the elimination of maltose forces S. bovis to utilize the starch-dye complex as the major substrate. PSE agar plus amylose azure gave the sharpest zones, but the zones were not as large as those produced on KFO1 agar. Modified PSE agar did not produce zones as large as would be expected when esculin is eliminated from the medium. Amylopectin azure gave sharper zones of equal size compared to amylose azure in modified KF Streptococcus agar, but smaller and not as clear zone when PSE agar is employed. This would tend to confirm the conclusion reached by Kohlhoff (1973) that the high molecular weight starches are not broken down as fast as the low molecular weight starches. Figure 4 shows the hydrolysis zones produced by S. bovis in PSE plus amylose azure agar.

Starch-Dye-Agar Overlay Method

The technique used in this investigation was developed in an effort to increase the size and sharpness of the starch-hydrolysis zones produced by S. bovis. It can be reasoned that a thin layer

Table 4. Summary of starch-hydrolysis zones produced by Streptococcus bovis using the starch-dye-medium method.[a]

Medium	Sugar (%)	Starch-Dye (0.2%)	Incubation Time 24 Hr Starch Zone Size (mm)	24 Hr Starch Zone Sharpness	48 Hr Starch Zone Size (mm)	48 Hr Starch Zone Sharpness[b]
Modified M-Enterococcus	Raffinose 0.2	Amylose Azure	2.0	+	3.0	+
Modified KF Streptococcus	Maltose 2.0	Amylose Azure	2.0	+	3.0	+
Modified KF Streptococcus	Maltose 2.0	Amylopectin Azure	2.0	++	3.0	++
KF01	none	Amylose Azure	4.0	++	7.0	++
Pfizer Selective Enterococcus	Esculin 0.1	Amylose Azure	3.0	+++	6.0	+++
Pfizer Selective Enterococcus	Esculin 0.1	Amylopectin Azure	2.5	++	4.0	++
Modified PSE	none	Amylose Azure	2.0	++	5.0	++

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2.

[b] A rating of (+) is the lowest rating and (+++) the highest rating.

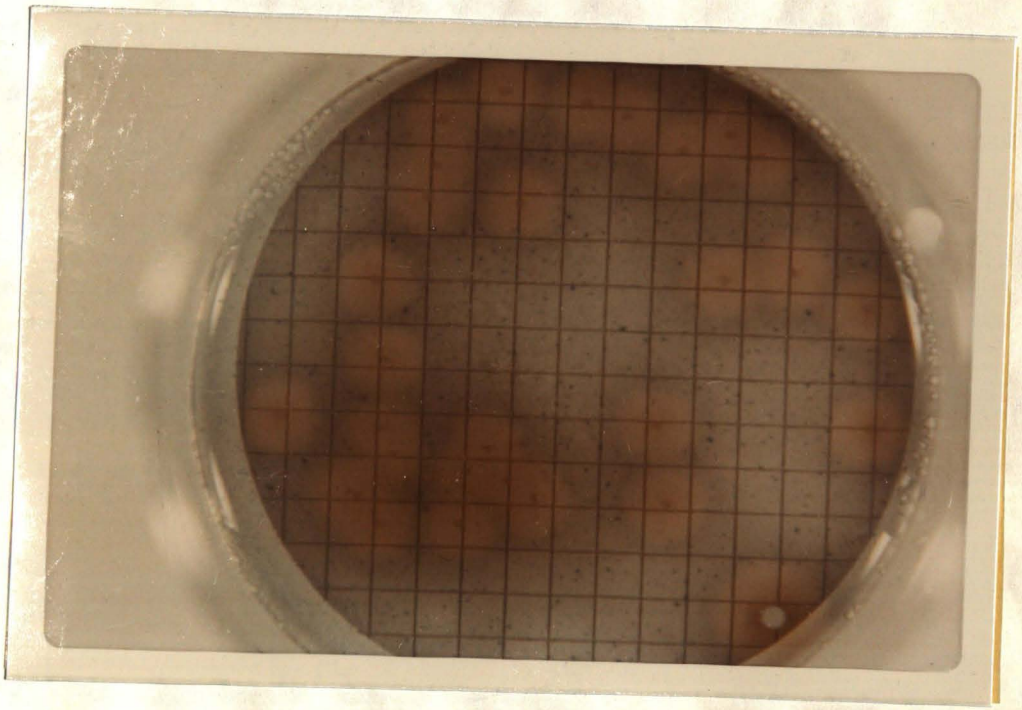


Figure 4. Zones of starch hydrolysis produced by Streptococcus bovis on a membrane filter layered with Pfizer Selective Enterococcus (PSE) agar with amylose azure as an indicator.

of a starch-dye-agar complex should give a larger zone than a thicker layer of a starch-dye medium complex when placed upon a membrane filter. PSE agar with an amylose azure overlay gave the largest and sharpest zones (Table 5). The zones produced in KFO1 agar are not as large or distinct as those produced in modified KF Streptococcus agar. Modified PSE agar did not support the growth of S. bovis. It may be speculated from these results that the removal of maltose from modified KF Streptococcus agar and esculin from PSE agar makes these media inhibitory to S. bovis.

The zones of hydrolysis produced using this method are no larger or clearer than those produced by the starch-dye medium method (Table 4).

Selectivity

Koupal (1970) was able to distinguish S. bovis and atypical S. faecalis from the other fecal streptococci by their ability to hydrolyze starch. Even though his medium and method were less inhibitory to S. bovis, they were not selective for the starch hydrolyzing fecal streptococci.

The removal of esculin in modified PSE agar and maltose in KFO1 agar were made so that the effects of sugar depletion could be measured with respect to selectivity. The starch-dye methods of isolation were to be compared in conjunction with these media on various fecal streptococci.

Trial runs with Streptococcus faecalis ATCC 8043 indicated that this was an atypical starch-hydrolyzing strain. Identification

procedures including starch hydrolysis and raffinose fermentation (Ramadan and Sabir, 1963) confirmed this observation. This strain was used as an atypical S. faecalis in this study.

KFO1 agar was able to support the growth of all of the fecal streptococci tested using the starch-dye medium method (Table 6). S. bovis and atypical S. faecalis exhibited the same amount of starch hydrolysis. S. faecalis and S. equinus, a weak starch-hydrolyzing fecal streptococci, did not hydrolyze the starch-dye complex. Modified PSE supported the growth of only S. bovis and atypical S. faecalis. The zones of hydrolysis produced by S. bovis are larger and more distinct than atypical S. faecalis on modified PSE agar, but this observation could not be used to differentiate between these two in a mixture of young colonies. It may be speculated from the results that modified PSE agar using the starch-dye medium method of indicating starch hydrolysis could be used to indicate ruminant pollution since atypical S. faecalis is also associated with the rumen (Mann et al., 1954; Cooper and Ramadan, 1955; and Ramadan and Sabir, 1963).

Table 7 provides a summary of data concerning the growth of various fecal streptococci in media lacking a sugar using the starch-dye-agar overlay method of isolation. Growth can be observed on KFO1 agar by all of the fecal streptococci tested although only S. bovis and atypical S. faecalis could hydrolyze the starch-dye complex. The zones of hydrolysis are nearly equal in size and appearance so they could not be used to distinguish S. bovis

Table 5. Summary of starch-hydrolysis zones produced by Streptococcus bovis using the starch-dye-agar overlay method.[a]

Medium	Sugar (%)	Starch-Dye (0.2%)	Incubation Time 24 Hr Starch Zone Size (mm)	24 Hr Starch Zone Sharpness	48 Hr Starch Zone Size (mm)	48 Hr Starch Zone Sharpness[b]
Modified M-Enterococcus	Raffinose 0.2	Amylose Azure	2.0	+	3.0	+
Modified KF Streptococcus	Maltose 2.0	Amylose Azure	2.0	+	3.0	+
Modified KF Streptococcus	Maltose 2.0	Amylopectin Azure	2.0	++	3.0	++
KF01	none	Amylose Azure	1.5	+	3.0	+
Pfizer Selective Enterococcus	Esculin 0.1	Amylose Azure	3.0	+++	6.0	+++
Pfizer Selective Enterococcus	Esculin 0.1	Amylopectin Azure	2.5	+++	5.0	+++
Modified P3E	none	Amylose Azure	0.0	none	0.0	none

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2.

[b] A rating of (+) is the lowest rating and (+++) the highest rating.

Table 6. Summary of plate counts and starch-hydrolysis zones produced by fecal streptococci on various media using the starch-dye-medium method.[a]

Medium	Sample Source	Starch Zone Size (mm)	Starch Zone Sharpness[b]	No. of Bacteria/ml (X 10^8)
KF01	Known *S. bovis*	7.0	+++	10
KF01	Known *S. faecalis*	0.0	none	8
KF01	Known atypical faecalis	6.0	+++	16
KF01	Known *S. equinus*	0.0	none	5
Modified PSE	Known *S. bovis*	5.0	++	16
Modified PSE	Known *S. faecalis*	0.0	none	0
Modified PSE	Known atypical faecalis	2.0	++	6
Modified PSE	Known *S. equinus*	0.0	none	0

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2; time, 48 hr; 0.2% amylose azure incorporated into the media to visualize hydrolysis.

[b] A rating of (+) is the lowest rating and (+++) the highest rating.

Table 7. Summary of plate counts and starch-hydrolysis zones produced by fecal streptococci on various media using the starch-dye-agar overlay method.[a]

Medium	Sample Source	Starch Zone Size (mm)	Starch Zone Sharpness[b]	No. of Bacteria/ml (X 10^8)
KF01	Known *S. bovis*	2.0	+	12
KF01	Known *S. faecalis*	0.0	none	11
KF01	Known atypical faecalis	1.5	+	10
KF01	Known *S. equinus*	0.0	none	15
Modified PSE	Known *S. bovis*	0.0	none	0
Modified PSE	Known *S. faecalis*	0.0	none	0
Modified PSE	Known atypical faecalis	0.0	none	0
Modified PSE	Known *S. equinus*	0.0	none	0

[a] Incubation conditions were: temperature, 35 C; atmosphere, 75% N_2, 25% CO_2; time, 48 hr; 0.2% amylose azure-1.0% agar overlay used to visualize starch hydrolysis.

[b] A rating of (+) is the lowest rating and (+++) the highest rating.

from atypical S. faecalis in a water sample. Growth can not be observed by any of the fecal streptococci tested in modified PSE agar. It may be that the elimination of esculin makes this medium nutritionally inadequate for the growth of fecal streptococci by changing the chemistry of the medium. However, growth of S. bovis and atypical S. faecalis was observed when these plates were removed from anaerobic conditions after 48 hr and then incubated aerobically.

General Observations

A rapid method to detect the presence of Streptococcus bovis in water could be used to indicate recent ovine or bovine pollution.

The results of this investigation concur that the most selective method of enumerating S. bovis should include the starch-dye medium membrane filtration technique described in the Materials and Methods section and modified PSE agar employing amylose azure as an indicator. The modified PSE agar contains no esculin, and 2,3,5-triphenyl tetrazolium chloride (TTC) is added at a concentration of 1.0 ml 1.0% TTC per 100 ml of medium to color the colonies.

Even though the main objective of this study was to determine the best method of rapid detection of S. bovis, further work must be done to make this method more selective for the enumeration of this organism. The effects of aerobic incubation and chemical alterations of the medium on amylase should be analyzed in an effort to make this method more selective for S. bovis while still inhibiting the growth of the other starch-hydrolyzing streptococci. This improvement may

eliminate the need for biochemical characterization of starch-hydrolyzing isolates.

CONCLUSIONS

1. Pfizer Selective Enterococcus (PSE) agar and modified KF Streptococcus agar are more suited for isolation of Streptococcus bovis than modified M-Enterococcus agar when using membrane filter techniques.
2. PSE agar gave the largest zones of starch hydrolysis using the starch-agar-overlay isolation method.
3. KFO1 agar and PSE agar incorporating amylose azure as an indicator gave large zones when using the starch-dye-medium method of isolating S. bovis.
4. The starch-dye-agar overlay method is no better than the starch-dye-medium method for isolating S. bovis.
5. The elimination of maltose in KFO1 agar does not make this medium more selective for S. bovis when using the starch-dye-medium or starch-dye-agar overlay methods of enumerating S. bovis.
6. Modified PSE agar using the starch-dye-medium membrane filter technique is selective for the amylolytic fecal streptococci.

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